Other Virological Mechanisms

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# Disclosure

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<th>Gilead Sciences Inc</th>
<th>Arrowhead Research Corp</th>
<th>Spring Bank Pharmaceuticals, Inc.</th>
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<th>AusBio Ltd</th>
<th>Janssen (J&amp;J)</th>
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<td>Consulting Fees (eg. Advisory Boards)</td>
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Hepatitis B: Global Burden

- 240 - 350 million people living with CHB globally

- 786,000 attributable deaths from hepatitis B annually in 2010; 1.3 million from viral hepatitis B & C collectively (GBD 2010)

- Viral hepatitis was the 9th ranked cause of human death; similar numbers of deaths to HIV, malaria and TB (GBD 2010)

- Without appropriate management, 15-25% of people with CHB will develop advanced liver disease &/or HCC

- Liver cancer is the 2nd most common cause of cancer death globally
  - GBD report 2013

Time 20-30 years

Normal  Cirrhosis  Cirrhosis  HCC
Mechanism(s) by Which HBV Causes Liver Cancer are Unresolved

- high levels of HBV replication
- persistent HBsAg protein expression
- Persistent HBeAg positivity
- truncated HBV proteins (preS deletions)
- BCP (HBx) variants
- integrated HBV DNA
- genotype (genotype C is overall the most oncogenic, whilst A1 and F1 can result in rapid progression to HCC)
- splicing/novel proteins  
  
  (Bayliss et al, J. Hepatol 2013)
Role of Splicing in HBV Life-Cycle Unclear

- In HIV, splicing is essential for expression of numerous viral proteins (Tat, Rev, Env etc).
- Splicing is not required for HBV replication.
- HBV spliced RNA can be packaged and reverse transcribed \((\text{Terre et al. J Virol 1991; 65: 5539})\)
- about 1-10% of the virion DNA populations contain spliced genomes
- splice mutants are replication defective due to intron removal generating truncated viral proteins \((\text{Sommer et al Virology, 1997;239:402})\)
- splice transcripts do not encode Pol, but are “replicated” by WT Pol in trans
- Secreted splice RNA recently identified in patient serum \((\text{Espiritu..Lam, AASLD, 2016})\)
- Clinical, virological and pathological significance now emerging.
Natural History and HBV Splicing

- Splicing of HBV is a common event during chronic infection, occurring in over 80% of patients
  - HBV splicing levels change dramatically over time, suggesting a highly dynamic process
  - Higher HBV viral load results in increased HBV splicing
  - Splicing increases by approximately 0.1% each year prior to the development of HCC
  - Asian HBV genotypes (B and C) have significantly greater levels of HBV splicing than European genotypes (A and D)
  - Following diagnosis of HCC it becomes increasingly difficult to interpret any patterns from HCC splicing
Multiple Splice Variants Have Been Identified

The most common transcript (Sp1) is a 2.2kb molecule that encodes precore/core, X and a novel protein in the hepatitis B splice protein (HBSP) \cite{Soussan2008}.
Hepatitis B Splice Protein (HBSP)

Chronic Hepatitis B
- 40% of patients have anti-HBSP
- associated with HBeAg-positivity and high VL
- associated with liver fibrosis
- induces apoptosis without cell cycle block in vitro
How Might HBV Splicing be Contributing to HCC #1?

- Splicing removes cis-acting sequence (hM) which promotes circular DNA, leading to double stranded linear DNA (Abraham et al. 2008)

- Double stranded linear DNA integrates into the host genome, and integration is strongly associated with liver cancer

Double-stranded linear (DSL) DNA is the dominant template for integration into the host genome.
ORFs of HBV dsIDNA Form

PreC/Core (1816-2454)
PreS1/S2/S (2850-837)
Pol (2309-1625)
X(1376-1840)

DR1
S1 Pro
S2 Pro
EN1/ XPro
EN2/ CPro

How Might HBV Splicing be Contributing to HCC #2?

- Alexander cells derived from HCC encode many integrated splice variants
- Secreted splice variants associated with advanced liver disease \((\text{Soussan et al. 2008})\)
- HBSP \((\text{Soussan et al., 2008})\) associated with increased cell proliferation and liver disease \((\text{Redelsperger, 2012})\)
- Recently shown that splicing generates oncogenic chimeric integrants in setting of hepatocellular carcinoma \((\text{Chiu et al., J. Hepatol. 2016})\)
2013 Pilot Study

• Serum was obtained from CHB patients with and without HCC

• HBV DNA was extracted and wt and SpDNA quantified by real time PCR, using our published method (Preiss et al., Hepatology, 2008; independently validated by Redelsperger et al., 2012)

• Analysed 62 serum samples from patients with HCC (n=32) and 157 samples from patients with chronic HBV infection alone (n=107)
Splice Variants in Serum were Associated with Liver Cancer

- Patients with liver cancer had significantly higher median Sp/wt HBV levels than those with chronic HBV infection alone (p= 0.001)
- Splice DNA increased prior to HCC diagnosis (1-3 years)

(Bayliss et al. J. Hepatol. 2013)

- Higher levels of Sp DNA in genotype B/C vs A/D
- Small pilot study. Does this finding hold up in larger cohorts?
The REVEAL Study

- Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-Hepatitis B Virus (REVEAL-HBV) study.
- Taiwanese study of 3653 male and female participants who were seropositive for HBsAg and seronegative for anti-HCV.
- The REVEAL study showed that the greatest risk factors for liver cancer were:
  - High viral load
  - HBeAg positivity
  - HBV genotype C

Are secreted splice variants predictive of HCC in this large Asian cohort?

Cohort Analysed

- Pre-diagnosed samples closest to HCC diagnosis (median time 2.5 years prior) of 152 incident HCC cases and matching samples from 378 age- and sex-matched non-HCC controls
- Earlier samples from 79 HCC cases and 33 controls were also assayed to examine long-term trajectories of spHBV levels, leading up to HCC
- DNA extracted in Taiwan and sent blinded to VIDRL
- Blinded results sent back to Taiwan

Prof H-I. Yang
Splice Variants Were Strongly Associated With Liver Cancer

- Logistic regression analysis was performed to examine the association between spHBV levels and HCC adjusted for other risk factors, and to calculate odds ratio (OR) with 95% confidence interval (CI)

- Statistical analysis performed by Prof. H-I Yang in Taiwan
Splice Variants Were Strongly Associated With Liver Cancer

- After adjustment for other liver cancer-related risk factors, subjects with spliced HBV DNA level \( \geq 10\% \) were 3.3 times more likely to develop liver cancer.
- Serum spHBV levels were higher in patients with older age, HBeAg seronegativity, genotype B, and precore mutant, and were negatively correlated with serum levels of HBV DNA and HBV surface antigen (HBsAg).
- The strongest association was identified for spHBV of 20% by Youden’s index analysis. The Odds ratio of developing HCC was 23.3 (P<.0001).
- Risk of HCC for patients with spHBV \( \geq 20\% \) raised to extremely high levels when combined with male gender, elevated HBV DNA and \( \alpha \)-fetoprotein, HBV genotype C, or liver cirrhosis.
Splice Variants Were Strongly Associated With Liver Cancer

• Most HCC patients who had elevated spHBV levels occurred up to 5 years prior to HCC diagnosis. The adjusted odds ratio of developing HCC within 5 years for spHBV ≥20% was 32.8 (P<.0001)
Clinical Implications

- Serum spHBV was an independent predictor of HCC development in the 5-year lead up to diagnosis

- Patients with serum spHBV levels above 20% should be checked further, especially for those who carried other risk factors
Conclusion

- Splice variants are associated with liver cancer and are more abundant and varied than previously appreciated.

- The mechanism is unresolved, but are likely to contribute to liver cancer through increased DNA integration (via DSL DNA), or expression of novel proteins such as HBSP.

- What is happening in the liver and in the serum?
HBV Transcripts Differ Between HBeAg+ and HBeAg- Chimps

PacBio Single Molecule Real-Time (SMRT) Sequencing

S transcripts in HBeAg- chimps often lack target sites for ARC-520

Splice Variants Were Strongly Associated With Liver Cancer

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? Screening for Serum Spliced HBV DNA
HBV RNA from Hepatitis B Patient Sera Contains Significant Amounts of Encapsidated Spliced HBV RNA Variants (2)

- HBV RNA is secreted within virus-like particles consist of envelope and capsid
- Extracellular HBV RNA particles contain pgRNA and spliced RNA variants
- HBV CAM blocks production of pgRNA and spliced RNA containing particles
- 3 new spliced variants identified

Espiritu C, et al. AASLD 2016, Boston. #17
Mechanism of RNA Interference (RNAi)

**Natural Process of RNAi**

1. **dsRNA** is cleaved by **dicer**.
2. **siRNAs** are produced.
3. **siRNAs** undergo **strand separation**.
4. The **RISC** complex forms via **complementary pairing**.
5. **RISC** cleaves the **mRNA**.
6. **mRNA degradation** leads to **cleaved mRNA**.

**Therapeutic Gene Silencing**

- **Synthetic siRNAs** are used to target specific genes.
Sp1 Encodes a Novel Protein- HBSP

3.6 kb pregenomic RNA

5’ Cap | Splice site | PreS1 | PreS2 | S | Splice site | X | poly(A) 3’

2.2 kb singly spliced RNA is a transcription template for Precore/core, X, and HBSP

5’ Cap | Precore/Core | -1 | Truncated S | X | poly(A) 3’

-1 | HBSP | Truncated Pol
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